

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE: 433

Acute Inhalation Toxicity - Fixed Concentration Procedure

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress and animal welfare considerations. The original acute inhalation Guideline 403 was adopted in 1981. Development of an Inhalation Fixed Concentration Procedure (FCP) was considered appropriate, following adoption of the revised oral FDP, OECD Guideline 420 in December 2001. This FCP guideline will allow the use of a series of fixed concentrations for the determination of acute inhalation toxicity in only one sex (usually females).
2. Traditional methods for assessing acute toxicity use death of animals as the sole endpoint. In 1984, a new approach to acute toxicity testing was suggested by the British Toxicology Society based on the administration of test material at a series of fixed dose levels (1). This approach avoided using death of animals as either an exclusive or an intended endpoint by incorporating evident clinical signs of toxicity at one of a series of fixed dose levels, as an endpoint on which to base classification of the test material. This approach is also taken for this guideline. In agreement with the OECD Guidance Document on Humane Endpoints (2) refinements are introduced in order to minimise any suffering and distress by the animals and, to the extent feasible, reduce the number of animals used. The statistical properties of the FCP have been evaluated using mathematical modelling (3).
3. Guidance on the conduct and interpretation of the Inhalation FCP can be found in the Guidance Document on Acute Inhalation Toxicity Testing (4).
4. Definitions used in the context of this Guideline are set out in Annex 1.
5. The method provides information on the hazardous properties and allows the substance to be ranked and classified according to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for the classification of chemicals which cause acute toxicity (5).

INITIAL CONSIDERATIONS

6. All available information on the test substance should be considered by the testing laboratory prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physico-chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; available (Q)SAR data and toxicological data on structurally related substances; the anticipated use(s) of the substance and the potential for human exposure. This information will assist in the selection of an appropriate starting concentration.

PRINCIPLE OF THE TEST

7. It is a principle of the method that only moderately toxic concentrations are used, and that administration of concentrations that are expected to be lethal should be avoided. Also, concentrations that are expected to cause marked pain and distress, due to corrosive¹ or severely irritant actions, should not be administered. Moribund animals, or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate OECD Guidance Document (2).

8. Groups of animals of a single sex are exposed for a short period of time to the test substance in a stepwise procedure using the appropriate fixed concentrations for vapours, dusts/mists or gases as set out in Annex 2. The initial concentration level is selected on the basis of a sighting study at the concentration expected to produce clear signs of toxicity without causing severe toxic effects or mortality. Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document on humane end-points (2). Further groups of animals may be tested at higher or lower fixed concentrations, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the concentration causing evident toxicity or no more than one death is identified, or when no effects are seen at the highest concentration or when deaths occur at the lowest concentration.

DESCRIPTION OF THE METHOD

Selection of animal species

9. The preferred rodent species is the rat, although on occasion other rodent species may be used. Justification should be provided for the use of other rodent or non-rodent species. Normally females are used. This is because there is thought to be little difference in sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive (6). However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided.

10. Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of testing, should be between 8 and 12 weeks old and its weight should fall within an interval of $\pm 20\%$ of the average body weight recorded at the laboratory for the particular strain used.

Housing and feeding conditions

11. The temperature of the experimental animal room should be $22 \pm 3^\circ\text{C}$. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by concentration, but the number of animals per cage should not interfere with clear observations of each animal.

¹ Determined using a validated test method (e.g., TG430 or 431) or an acceptable prediction.

Preparation of animals

12. The animals are acclimatised to the laboratory conditions for at least five days prior to the start of exposure. Animals are randomly selected for use in the study and marked to provide individual identification.

Mode of exposure

13. Both head/nose-only and whole-body exposure techniques may be used. The head/nose-only exposure method minimises exposure or uptake by non-inhalation routes and allows testing of individual animals at high concentrations, as required for limit tests, without the need for large quantities of material. Further advantages include; ease of maintenance of a homogenous test atmosphere, less potential for test material instability (*e.g.*, reaction with excreta or humidity), and faster equilibration of the chamber atmosphere due to the smaller volume required. This technique does, however, require restraint of the animals throughout the exposure period, which is not necessary for whole-body exposures. The selected exposure model should be designed to minimise any pain, distress or suffering experienced by the animals, consistent with the scientific objective of the study (2).

Head/nose-only exposure technique

14. During exposure, the animals are exposed to the test substance in exposure tubes. The animal restraining tubes should not impose undue stress on the animal, should be constructed in such a way as to avoid hyperthermic stress for the animal and should make it impossible for the animal to avoid inhalation exposure. However, if a negative balance of air volumes supplied and extracted cannot be avoided, a dilution of test atmosphere by bias-airflow (via exposure tubes) should be prevented. The inhalation chamber should be operated in well ventilated chemical hoods. Maintenance of slight negative pressure inside the hood will prevent leakage of the test substance into the surrounding areas. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow which exceeds at least twice the respiration ventilation volume of all animals in the inhalation device. An adequate oxygen content of at least 19% and similar exposure conditions at each exposure port should be ensured. During the sampling of the test atmosphere, a significant disturbance of the airflow dynamics should be avoided. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same.

Whole-body exposure technique

15. The animals should be tested using inhalation equipment designed to sustain a dynamic air flow of approximately 12 to 15 air changes per hour. Other air flow rates may be useful to meet specific requirements imposed by the test compound. However, an adequate oxygen content of at least 19% and an evenly distributed exposure atmosphere should be ensured. As a general rule to ensure the stability of a chamber atmosphere, the total volume of the test animals should not exceed five per cent of the volume of the test chamber. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

Exposure conditions

16. A fixed duration of exposure of at least four hours, excluding equilibration time, is recommended. Other durations may be needed to meet specific requirements.

17. To establish suitable exposure concentrations, a technical trial test without animals is mandatory. It is technically difficult to generate test atmospheres to accurately meet specified fixed exposure

concentrations. Therefore, to prevent unnecessary repeat testing, a test atmosphere is considered acceptable for regulatory purposes if the mean of the test atmosphere samples is within $\pm 25\%$ of the concentration tested. In the case of potentially explosive test substances, care should be taken to avoid favourable conditions for explosions.

Particle size

18. As it is difficult to predict the most responsive region of the respiratory tract or the most harmful particle size, the particle size distribution of dusts and aerosols should be such that exposure of all regions of the tract can be achieved. An aerosol with a mass median aerodynamic diameter (MMAD) between 1 to 4 μm and a geometric standard deviation (GSD) in the range of 1.5 to 3.0 is recommended to ensure that comprehensive respiratory tract exposure occurs (7). In case a laboratory deviates from the recommended MMAD, an explanation and justification should be given. When using hygroscopic particles, efforts should be taken to generate hygroscopic materials with smaller particles sizes than would be required for most other chemicals to ensure that the swollen particle will still be within the 1 to 4 μm range.

Generation of test atmospheres

19. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration and respirability of the test substance in the atmosphere. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, the acute inhalation toxicity of the vehicle should be known. A concurrent vehicle (or any other) control group is not considered necessary for well characterised control vehicles. Particulate material may be subjected to mechanical processes to achieve the required particle size distribution, however, care should be taken not to decompose or alter the test substance, which should be analytically verified. Adequate care should be taken not to contaminate the test material.

MONITORING OF EXPOSURE CONDITIONS

Chamber airflow

20. The flow of air through the exposure chambers should be monitored continuously and recorded at least three times during each exposure.

Chamber temperature

21. The air temperature in the animal's breathing zone should be monitored continuously and recorded at least three times during each exposure. Ideally the temperature should remain within the range $22 \pm 3^\circ\text{C}$. Deviations from this range should be commented upon with an assessment of the effect, if any, on the outcome of the exposure.

Relative humidity

22. The relative humidity (RH) in the animal's breathing zone, for both the head/nose only and the whole body exposures, should be monitored continuously and recorded three times during each exposure where possible. The RH should ideally be maintained in the range of 30 to 70% but it is recognised that under certain circumstances this may either be unattainable (*e.g.*, when testing water based formulations) or may not be measurable due to interference by the test substance with the test method.

Concentration of test substance

23. Actual concentrations of the test substance should be measured in the breathing zone of the rats in both the head/nose only and the whole body exposures. During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable (see paragraph 17) and monitored continuously or intermittently depending on the method of analysis. If intermittent sampling is used at least five samples should be taken at approximately hourly intervals. For single component powder aerosols and liquids that are of extremely low volatility, gravimetric analysis is acceptable. When performing gravimetric sampling at the higher exposure concentrations used in these studies, care should be taken to calibrate the flow meter (or dry gas meter) used to determine sampled volume as a function of the pressure drop across the filter (based upon the relationship pressure x volume = constant). A calibration volume curve should be generated for each flow meter or dry gas meter used.

24. For aerosols of liquid formulations that can be evaporated to a constant weight, gravimetric analysis of the dried residue may be used. Appropriate extrapolation to calculate the weight of formulation should be applied to the gravimetric data. It is not necessary to analyse inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation; the grounds for this conclusion should be provided in the study report. If there is some difficulty in measuring chamber analytical concentration due to precipitation, non-homogenous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary.

25. Where gravimetric analysis is unsuitable and the test atmosphere contains more than one component, chemical analysis of the major active ingredient followed by extrapolation to the concentration of formulation may be acceptable but should be justified.

26. Whenever the test substance is a formulation, the analytical concentration should be reported for the total formulation and not just for the active ingredient.

Particle size distribution

27. The particle size distribution of the test aerosol should be determined at least twice during each 4-hour exposure. A range of sampling devices is suitable but the device selected should allow calculation of the MMAD (See paragraph 18). In the case of multi-component aerosols the principles given above for determination of concentration should be applied. Adequate information should be available within the testing facility to demonstrate that such samplers collect an atmospheric sample that is representative of the atmosphere to which the animals are exposed.

Nominal concentration

28. The nominal exposure chamber or exposure tube concentration should be determined by recording the amount of test material disseminated into the exposure chamber/tube during the generation period and dividing this by the total airflow through the chamber/tube during the same period.

PROCEDURE

Sighting study

29. The purpose of the sighting study is to allow selection of the appropriate starting concentration

for the main study. The test substance is administered to single animals in a sequential manner following the flow charts in Annex 2 for a period of at least four hours. The sighting study is completed when a decision on the starting concentration for the main study can be made, based on signs of evident toxicity or if a death is seen at the lowest fixed concentration.

30. The starting concentration for the sighting study is selected from the fixed concentration levels found in Annex 2 as a concentration expected to produce evident toxicity based, when possible, on evidence from existing data on the same chemical and/or structurally related chemicals. In the absence of such information, the starting concentration will be 10 mg/l, 1 mg/l or 2500 ppm for vapours, dusts/mists and gases, respectively.

31. A period of at least 24 hours will be allowed between the testing of each animal. All animals should normally be observed for at least one week.

32. In cases where an animal tested at the lowest fixed concentration level in the sighting study dies or exhibits clear clinical signs of toxicity, the normal procedure is to terminate the study and assign the substance to GHS Category 1 (as shown in Annex 2). However, if further confirmation of the classification is required, an optional supplementary procedure may be conducted, as follows. A second animal is tested at the lowest fixed concentration. If this second animal dies, then GHS Category 1 will be confirmed and the study will be immediately terminated. If the second animal survives, then a maximum of three additional animals will be tested at this concentration. Because there will be a high risk of mortality, these animals should be tested in a sequential manner to protect animal welfare. The time interval between exposure of each animal should be sufficient to establish that the previous animal is likely to survive. If a second death occurs, the testing sequence will be immediately terminated and no further animals will be tested. The classification will be as shown in Annex 2: Category 1 if there are two or more deaths (outcome A), or Category 2 if there is one death (outcome B).

MAIN STUDY

Numbers of animals and concentration levels

33. The action to be taken following testing at the starting concentration level is indicated by the flow charts in Annex 2. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed concentration or test at a lower fixed concentration. However, a concentration level, which caused death in the sighting study, will not be revisited in the main study, to protect animal from unnecessary suffering (see Annex 2). Experience has shown that the most likely outcome at the starting concentration level will be that the substance can be classified and no further testing will be necessary. When testing a descending series and 2-3 deaths are observed (within the scope of outcome A), then in the interests of animal welfare the test should be halted and the substance classified according to outcome C of the next concentration in the series.

34. A total of five animals of one sex will normally be used for each concentration level investigated, in addition to the single animal used in the sighting study.

35. The time interval between exposures at each level is determined by the onset, duration and severity of toxic signs. Exposure of animals at the next concentration should be delayed until there is confidence in the survival of the previously tested animals. A period of three or four days between the exposures at each concentration level is recommended to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, *e.g.*, in case of inconclusive response.

Limit Test

36. The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be non-toxic, *i.e.*, having toxicity only above regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

37. Using the normal procedure, a main study starting concentration of 20 mg/l, 5mg/l or 5000 ppm for vapours, dusts/mists and gases, respectively, followed by exposure of a further five animals at this level serves as a limit test for this guideline, if achievable. In some cases, as required by some regulatory authorities, testing up to the limit of GHS class 5 may be conducted. However, testing in animals in Category 5 ranges is discouraged and should only be considered when there is a strong likelihood that the results of such testing would have a direct relevance to the protection of human health (5).

Observations

38. During the exposure period the animals should be observed frequently. In addition, after exposure, careful clinical observations should be made at least twice on the day of exposure, or more frequently, when indicated by the response of the animals to the treatment, and at least once daily thereafter, for a total of 14 days, except where they are found dead. However, the duration of observation is not fixed but should be determined by the nature and time of onset of clinical signs and length of the recovery period. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for signs of toxicity to be delayed. All observations are systematically recorded, with individual records being maintained for each animal. Animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed without delay. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

39. Additional observations will be necessary if the animals continue to display clinical signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. If possible, a differentiation between local and systemic effects should be determined. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarised in the Humane Endpoints Guidance Document should be taken into consideration (2). Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed for animal welfare reasons. Care should be taken (*e.g.*, by using control animals exposed to air) when conducting examinations for clinical signs of toxicity that initial poor appearance and transient respiratory changes, resulting from the exposure procedure, are not mistaken for treatment-related effects.

Body weight

40. Individual weights of animals should be determined on the day of, or immediately prior to the administration of the test substance and at least weekly thereafter. At the end of the test surviving animals are weighed and then humanely killed.

Pathology

41. All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the initial exposure may also be considered because it may yield useful information.

DATA AND REPORTING

Data

42. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of effects of toxicity and reversibility, and necropsy findings.

Test Report

43. The test report should include the following information, as appropriate:

Test substance:

- physical nature, purity, and, where relevant, physico-chemical properties (including isomerisation);
- identification data and Chemical Abstract Services Registry Number, if known.

Vehicle

- justification for use of vehicle and justification for choice of vehicle (if other than water).

Test animals:

- species/strain used;
- microbiological status of the animals, when known;
- acclimatisation period;
- number, age and sex of animals (including, where appropriate, a rationale for use of males instead of females);
- source, housing conditions, historical data, diet etc.;

Test conditions:

- details of test substance preparation, including details of any procedures used to reduce the particle size of powders or to prepare solutions of the test substance;
- a description (preferably including a diagram) of the equipment used to generate the test atmosphere and to expose the animals to the test atmosphere;
- details of the equipment used to monitor chamber temperature, humidity and airflow;
- details of the equipment used to collect samples for determination of chamber concentration and particle size distribution;
- details of the chemical analytical method used and method validation (including efficiency of recovery of test substance from the sampling medium);

- details for time needed for equilibrium of exposure concentration before animal exposure;
- method of randomisation in assigning animals to test and control groups;
- details of food and water quality (including diet type/source, water source);
- the rationale for the selection of the starting concentration.

Results:

- tabulation of chamber temperature, humidity and airflow;
- tabulation of chamber nominal and actual concentration data;
- tabulation of particle size data including analytical sample collection data, particle size distribution and calculations of the MMAD and GSD;
- tabulation of response data and concentration level for each animal (*i.e.*, animals showing signs of toxicity including mortality, nature, severity, and duration of effects);
- individual weights of animals at the day of exposure, in weekly intervals thereafter, and at time of death or euthanasia; date and time of death if prior to scheduled euthanasia, time course of onset of signs of toxicity and whether these were reversible for each animal;
- necropsy findings and histopathological findings for each animal, if available.

Discussion and interpretation of results.

Conclusions.

LITERATURE

- (1) British Toxicology Society Working Party on Toxicity (1984). Special report: a new approach to the classification of substances and preparations on the basis of their acute toxicity. *Human Toxicol.*, 3, 85-92.
- (2) OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19. Available: : [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html]
- (3) Stallard, N. and Whitehead, A. (2002). Statistical evaluation of the fixed concentration procedure for acute inhalation toxicity assessment (manuscript).
- (4) OECD. Draft Guidance Document No. 39 on “Acute Inhalation Toxicity Testing”. Environmental Health and Safety Monograph Series on Testing and Assessment.
- (5) United Nations (UN)(2003). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), ST/SG/AC.10/30, UN New York and Geneva. Available: [<http://www.unece.org/trans/danger/publi/ghs/officialtext.html>]
- (6) Warbrick, E. V., Indans, I., Blackwell, M., Hardy, C. and Shepherd, N. (2002). The reduction and refinement of animal use in acute inhalation toxicity testing. *Toxicol.*, 192, 92.
- (7) SOT COMMENTARY, (1992). Recommendations for the Conduct of Acute Inhalation Limit Tests. *Fundam. Appl. Toxicol.*, 18, 321-327.
- (8) Van den Heuvel, M. J., Clark, D. G., Fielder, R. J., Koundakjian, P. P., Oliver, G. J. A., Pelling, D., Tomlinson, N. J. and Walker, A. P. (1990). The international validation of a fixed-dose procedure as an alternative to the classical LD₅₀ test. *Fd. Chem. Toxicol.*, 28, 469-482.

ANNEX 1

DEFINITIONS

Acute inhalation toxicity is the total of adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time to an airborne substance.

Aerosol: A suspension of solid or liquid particles in a suspension in a gas, as a foam, paste or powder or in a liquid state or in a gaseous state.

Concentration is expressed as weight of the test substance per unit volume of air, for vapours and dusts as mg/L and for gases as ppm (parts per million), in accordance with the UN GHS.

Dust: Solid particles formed from a substance or mixture, capable of being suspended in air. These particles may have irregular shapes with sizes ranging from sub-micrometer up to over 100 µm.

Evident toxicity is a general term describing clear signs of toxicity following the administration of a test substance, (see Van den Heuvel *et al.*, 1990 (8) for examples) such that at the next highest fixed concentration either severe pain and enduring signs of severe distress, moribund condition (criteria are presented in the Humane Endpoints Guidance Document (2)) or probable mortality in most animals can be expected.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): a system proposing the classification of chemicals according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people and the environment. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical-chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor. (See the Humane Endpoint Guidance Document (2) for more details).

Limit dose refers to a dose at an upper limitation on testing using 20 mg/l, 5 mg/l or 5000 ppm for vapours, dusts/mists and gases, respectively.

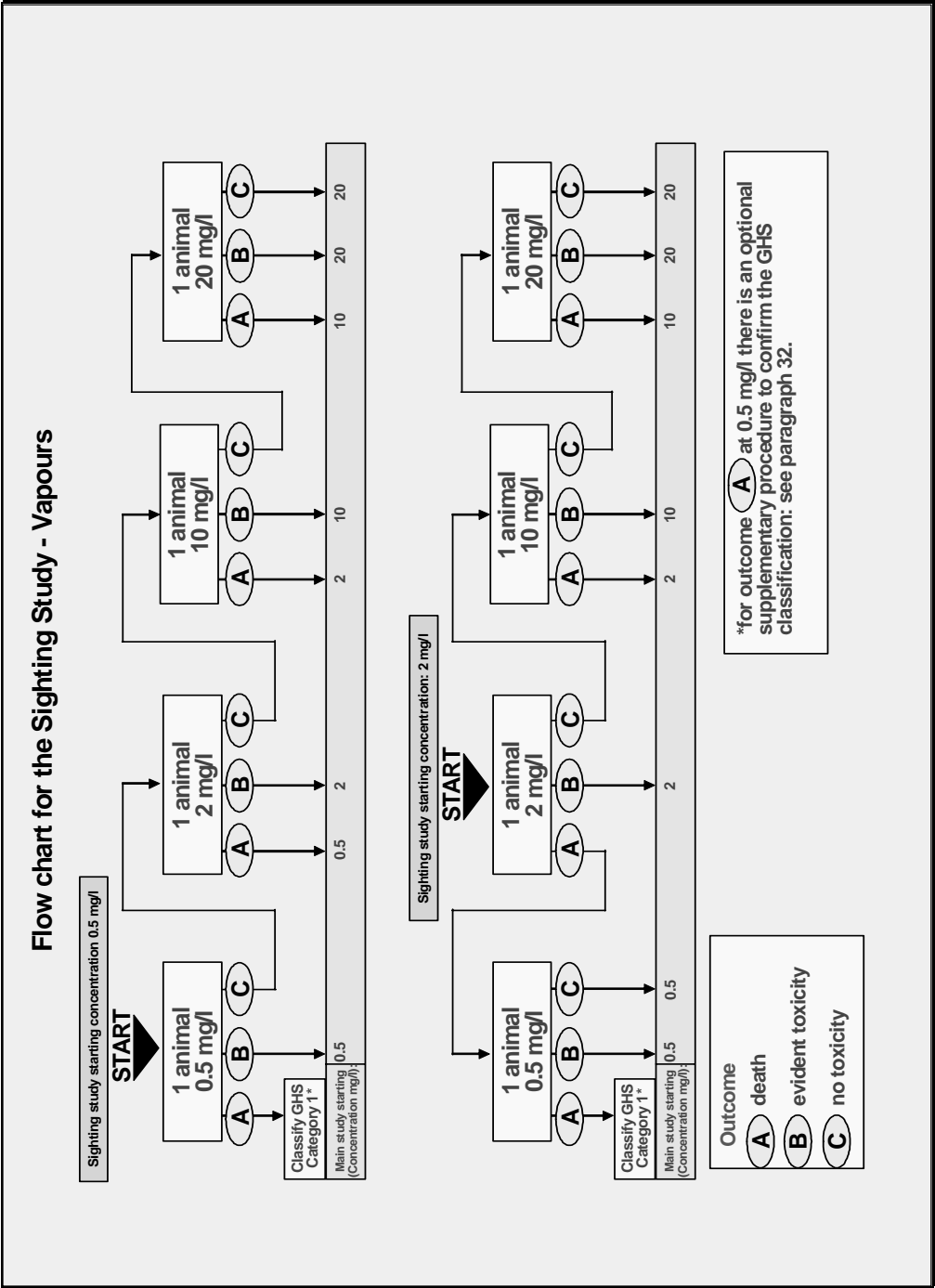
Mass median aerodynamic diameter (MMAD): The median aerodynamic diameter and, along with the geometric standard deviation, is used to describe the particle size distribution of any aerosol statistically, based on the weight and size of the particles. Fifty percent of the particles by weight will be smaller than the median diameter and 50 percent of the particles will be larger.

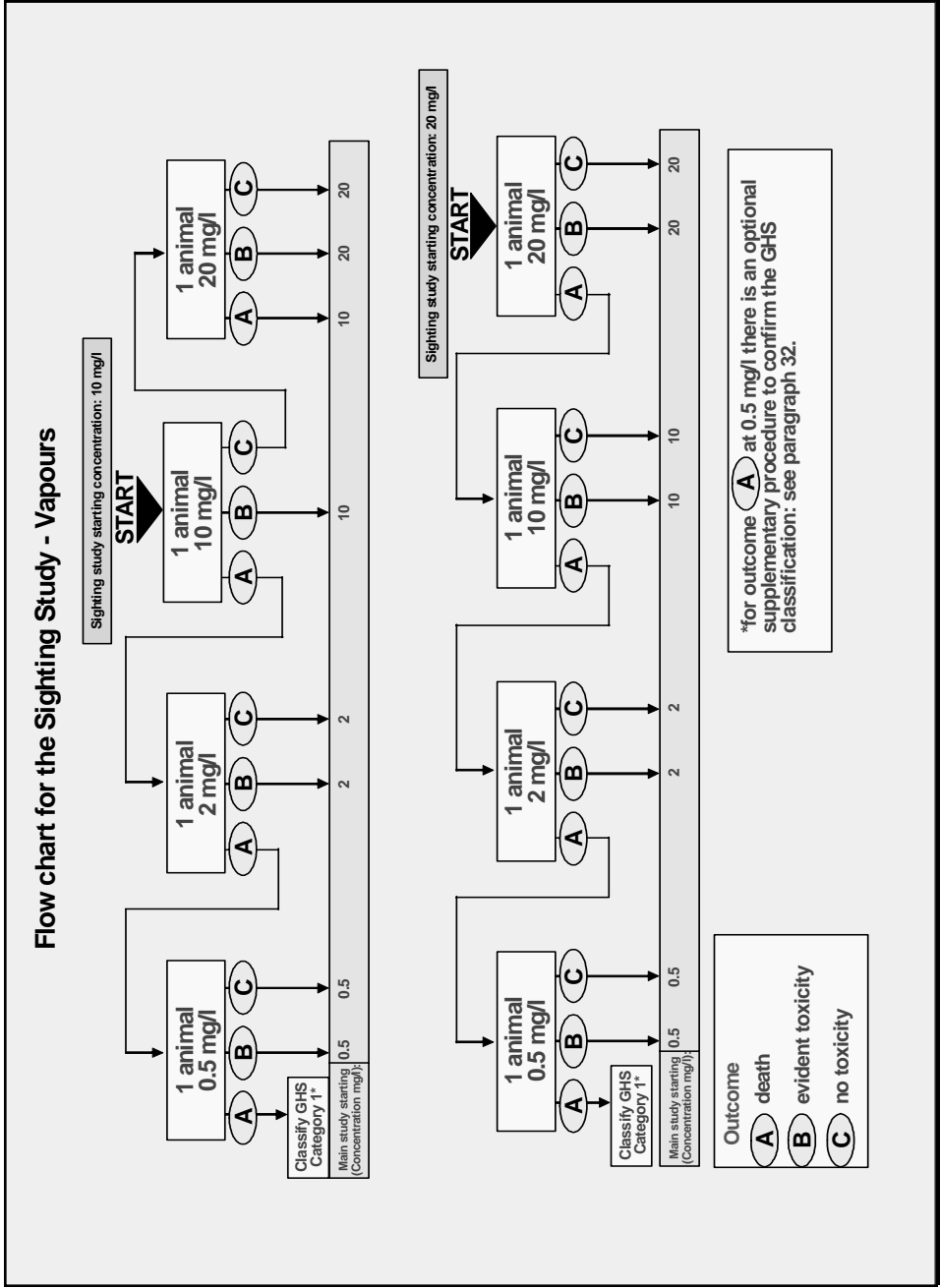
Mist: Finely divided liquid droplets of a substance or mixture suspended in air with sizes generally ranging from 2 to 100 µm. A mist can be formed by condensation of supersaturated vapours or by physical shearing of liquids, such as nebulization, atomisation, spraying or bubbling.

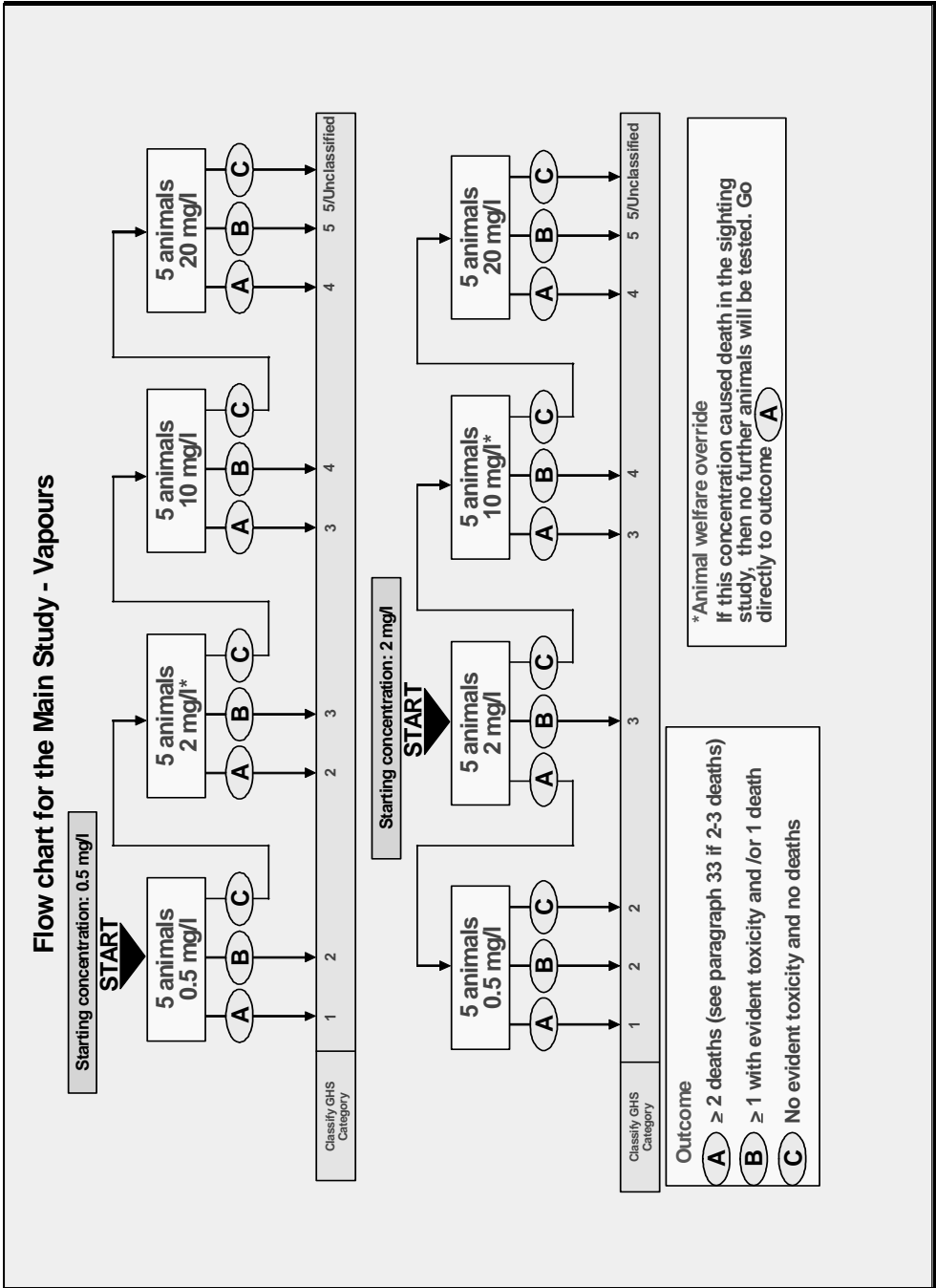
Moribund condition: being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document (2) for more details).

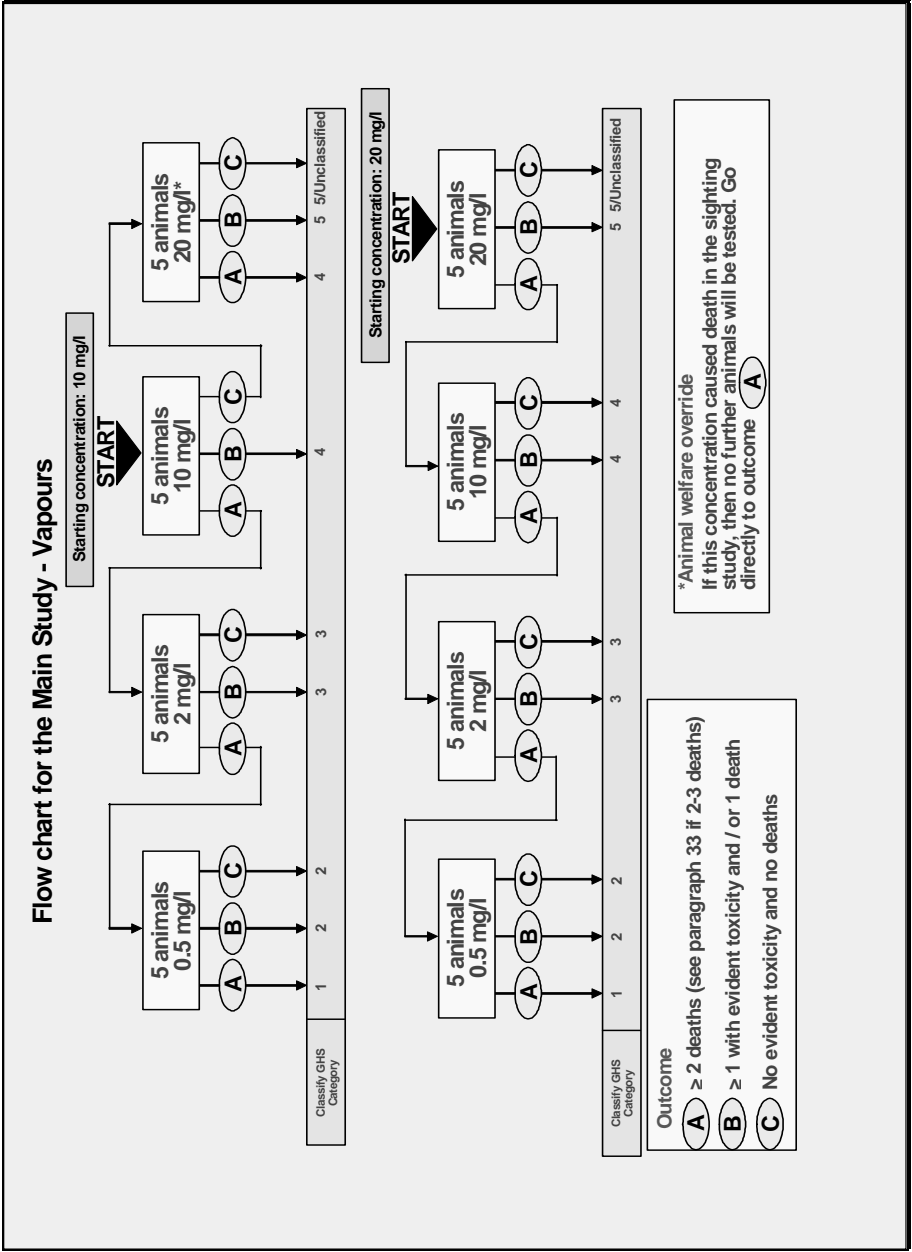
Vapour: The gaseous form of a substance or mixture which is normally in liquid or solid state at ambient conditions of temperature and pressure.

ANNEX 2

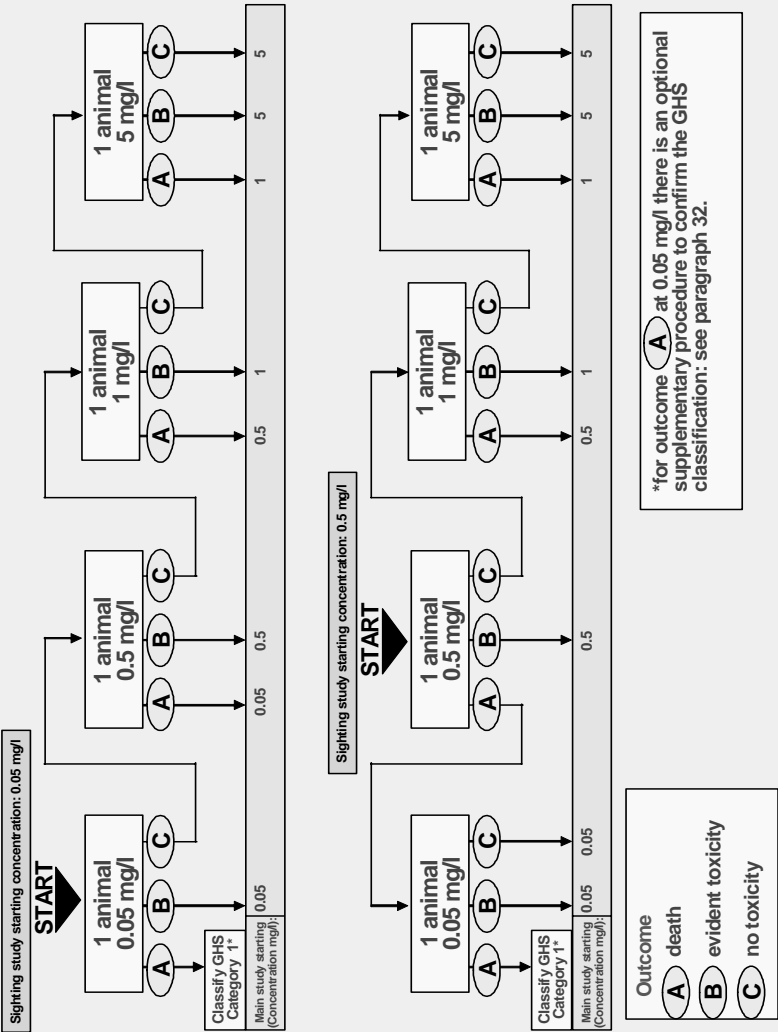


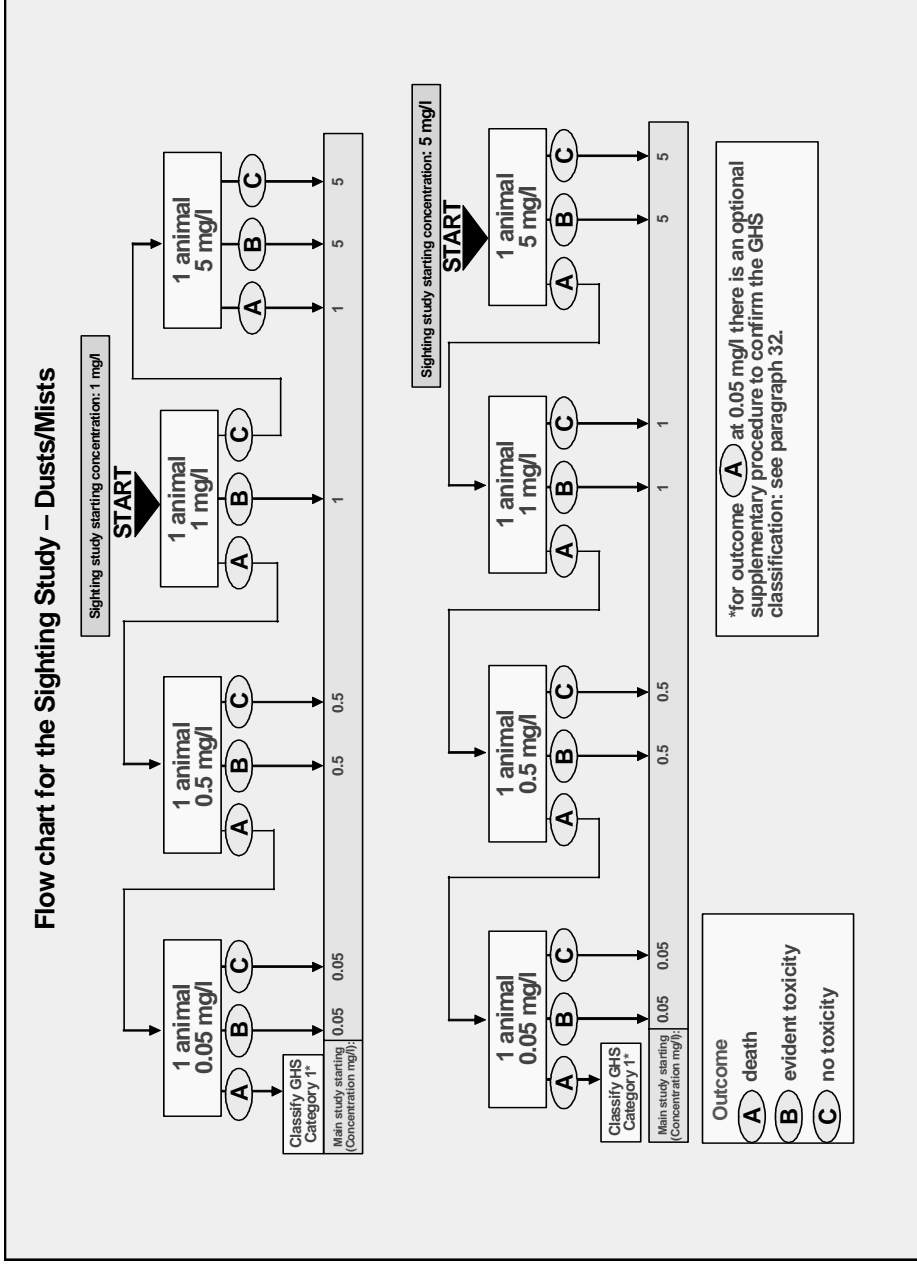


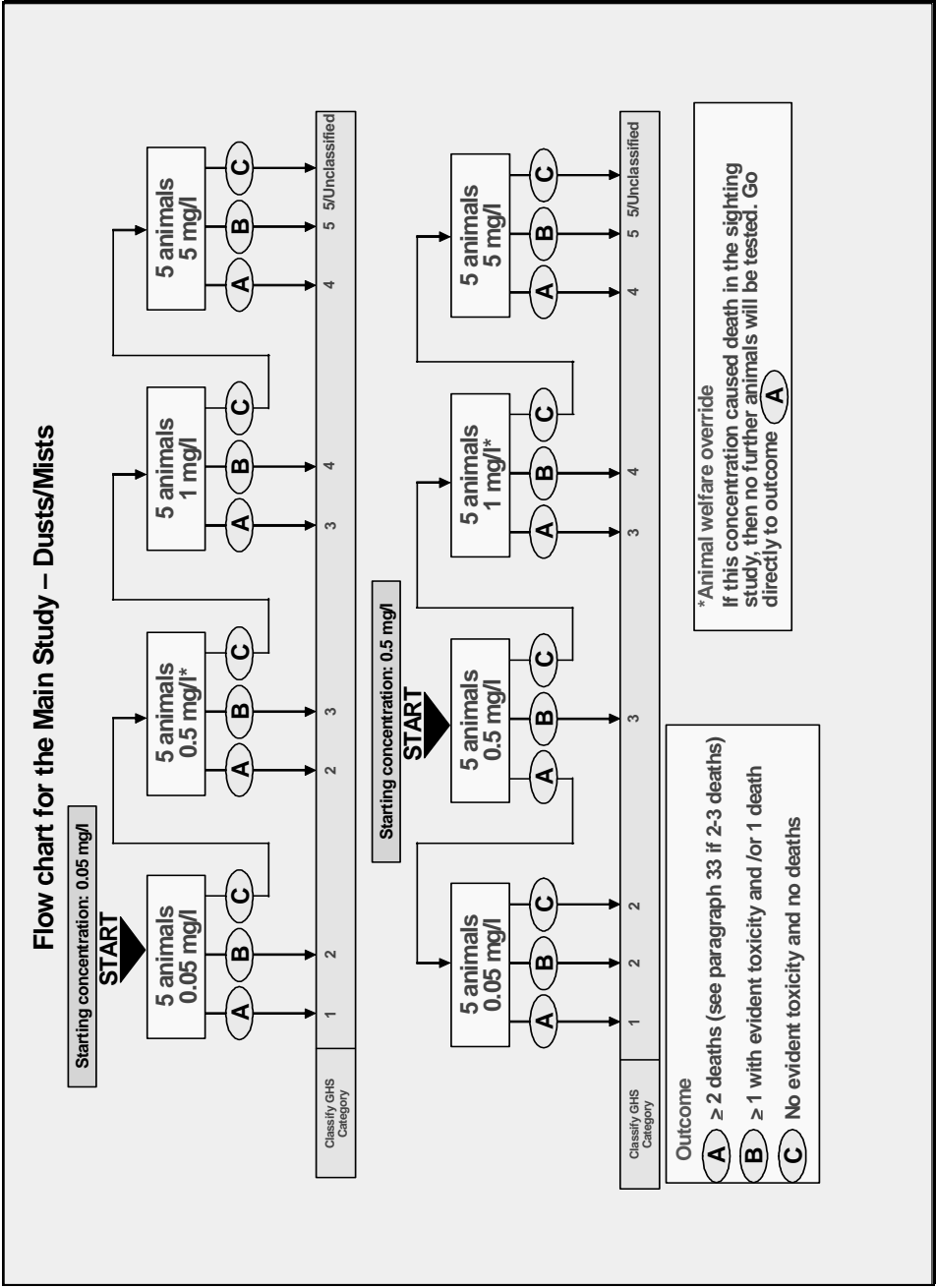


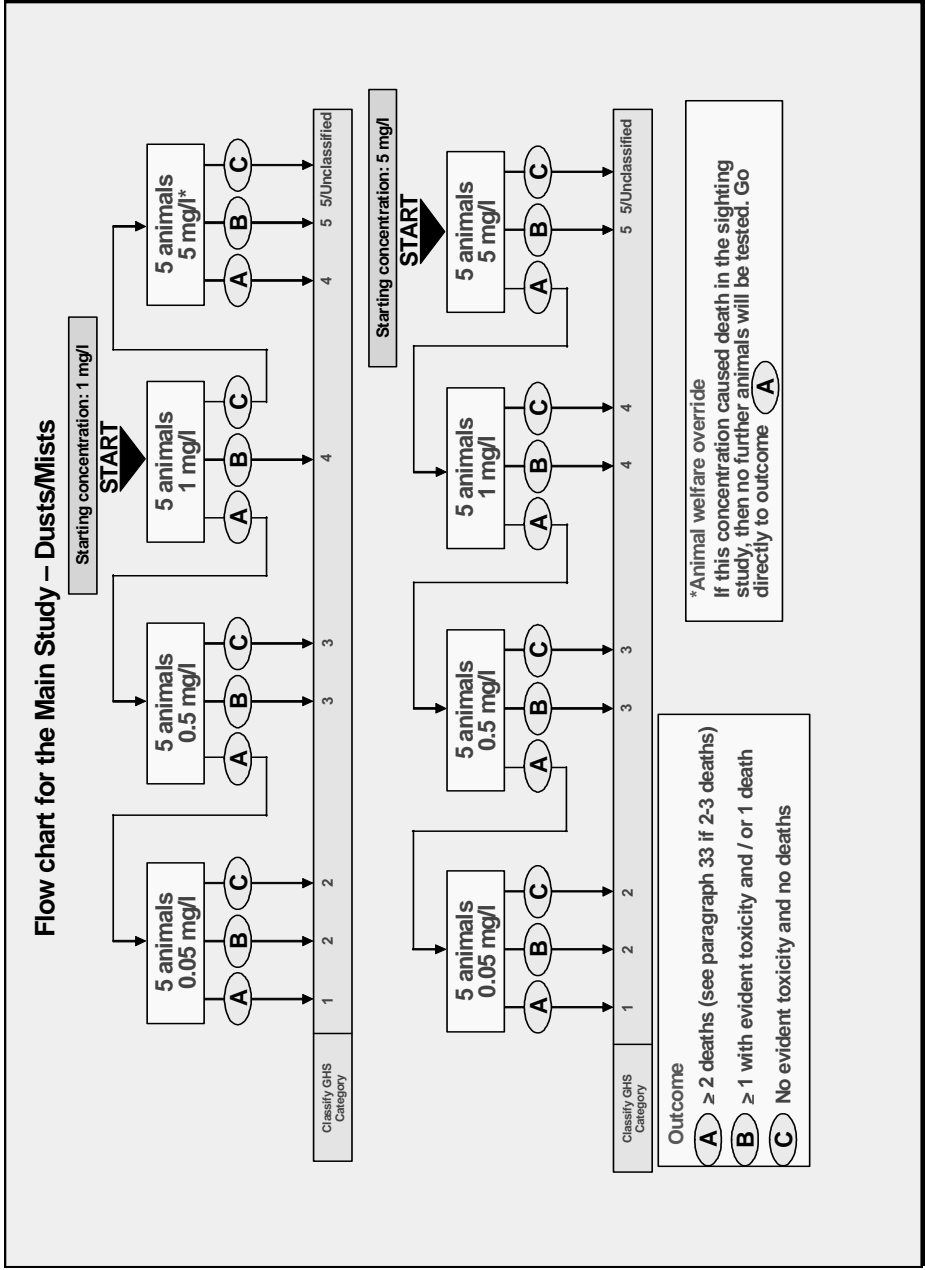


Flow chart for the Sighting Study – Dusts/Mists









Flow chart for the Sighting Study - Gases

